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Phosphorylation of TCF Proteins by Homeodomain-interacting Protein Kinase 2*

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Wnt pathways play essential roles in cell proliferation, morphogenesis, and cell fate specification during embryonic development. According to the consensus view, the Wnt pathway prevents the degradation of the key signaling component β-catenin by the protein complex containing the negative regulators Axin and glycogen synthase kinase 3 (GSK3). Stabilized β-catenin associates with TCF proteins and enters the nucleus to promote target gene expression. This study examines the involvement of HIPK2 (homeodomain-interacting protein kinase 2) in the regulation of different TCF proteins in *Xenopus* embryos *in vivo*. We show that the TCF family members LEF1, TCF4, and TCF3 are phosphorylated in embryonic ectoderm after Wnt8 stimulation and HIPK2 overexpression. We also find that TCF3 phosphorylation is triggered by canonical Wnt ligands, LRP6, and dominant negative mutants for Axin and GSK3, indicating that this process shares the same upstream regulators with β-catenin stabilization. HIPK2-dependent phosphorylation caused the dissociation of LEF1, TCF4, and TCF3 from a target promoter *in vivo*. This result provides a mechanistic explanation for the context-dependent function of HIPK2 in Wnt signaling; HIPK2 up-regulates transcription by phosphorylating TCF3, a transcriptional repressor, but inhibits transcription by phosphorylating LEF1, a transcriptional activator. Finally, we show that upon HIPK2-mediated phosphorylation, TCF3 is replaced with positively acting TCF1 at a target promoter. These observations emphasize a critical role for Wnt/HIPK2-dependent TCF phosphorylation and suggest that TCF switching is an important mechanism of Wnt target gene activation in vertebrate embryos.

Wnt signaling is an essential embryonic pathway that regulates cell fate determination, cell proliferation, and cell polarity. The Wnt pathway leads to the stabilization of β-catenin, which associates with TCF proteins to activate target genes (1, 2). Whereas the function of β-catenin in embryonic axis determination and Wnt signaling has been firmly established (3–5), genetic studies of TCF proteins reveal their diverse and complex roles in development (6–11). In a commonly accepted canonical model, TCFs bind Groucho/TLE corepressor proteins and inhibit target genes in the absence of a Wnt signal but associate with β-catenin and convert into activators after cell stimulation by Wnt proteins (12–14). In organisms, which possess a single TCF gene, such as *Caenorhabditis elegans* (*POP-1*) or *Drosophila* (*pangolin/dTCF*), TCF proteins play both negative and positive roles (15–17). By contrast, vertebrates carry four conserved TCF homologues, TCF1, LEF1, TCF3, and TCF4, which appear to have distinct functions and control different sets of targets at different times during development (7). Despite the important roles for TCF proteins in the control of gene expression during development and disease (1, 2, 7), the mechanisms of their regulation are still poorly understood.

Accumulating evidence shows that TCF proteins can be phosphorylated in response to Wnt proteins, raising the question whether this phoshorylation is important for determining the outcome of signaling. For example, phosphorylation of *Xenopus* TCF3 by casein kinase 1, a critical player in Wnt signaling (18–20) was proposed to stimulate β-catenin binding (21). In *C. elegans*, the protein kinase LIT-1 triggers POP-1/TCF phosphorylation, leading to its nuclear export that is required to promote the endodermal fate during Wnt signaling (22–25). Similarly, vertebrate TCF proteins LEF1 and TCF4 can be phosphorylated in cultured cells by Nlk (Nemo-like kinase), a mammalian homologue of LIT-1 (26–30), but the *in vivo* significance of this phosphorylation has not been established.

Another family of nuclear protein kinases that have been implicated in Wnt signaling and could play a role in TCF regulation are homeodomain-interacting protein kinases (HIPK1–4) (31). HIPK2 is expressed in multiple mouse embryonic tissues, including the brain, the heart, the kidney, and the muscle (32), and functions in transcriptional regulation, cell growth, and apoptosis (33, 34), presumably by activating p53 (35–37) or c-Jun N-terminal kinase (38). Embryos from mice lacking both *HIPK1* and *HIPK2* genes exhibit severe exencephaly with anterior neural tissue overgrowth and die between embryonic days 9.5 and 12.5 (39). HIPK2-mediated phosphorylation promotes proteasome-dependent degradation of C-terminal binding protein (41) and attenuates Groucho repressive activity (40). The HIPK2-Nlk complex was demonstrated to phosphorylate and degrade c-Myb in response to Wnt1 (42). Other studies reported both positive and negative effects of HIPK proteins in Wnt/β-catenin signaling in mouse embryo fibroblasts (43, 44), *Drosophila* and *Xenopus* embryos (45, 46), but the underlying mechanisms have not been fully elucidated.

We have recently discovered that TCF3 is phosphorylated by HIPK2 in response to Wnt8 stimulation and identified the relevant phosphorylation sites critical for its function (47). Based on the conservation of some of these phosphorylation sites in LEF1, TCF3, and TCF4 but not in TCF1, we hypothesize that HIPK2 is involved in the phosphorylation of different TCF pro-

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TABLE 1
Primers for TCF constructs

XTCF1
For mutagenesis
FLAG, 5′-GCAGGGGTAACTCACAGAACTGCAGATGGATTACAAGGATGA CGACGATAAGCCCCAAATGAACAGCGCCG-3′
mLEF1
For subcloning
Forward, 5′-GGGAATTCAATGCCCCAACTTTCCGGA-3′
Reverse, 5′-GCTCTAGATCAGATGTAGGCAGCTGTCAT-3′
For mutagenesis
P2, 5′-GTACATGTCAAATGGGTCGCTAGCTCCACCCATCCCGAGGA-3′
P3, 5′-TCACGCGGTCCACCCGCTAGCCCCCCTCATCACCTACAGCGACG AGCACTTTGCTCCGGGATCCCACCCGTC-3′
XTCF4
For subcloning
Forward, 5′-GGAATTCAATGCCGCAGTTGAATGGC-3′
Reverse, 5′-CCGCTCGAGCTAATAAGCTTCCATCTG-3′

teins. To test this hypothesis, we examined the phosphorylation state of TCF family proteins and observed a similar regulation of LEF1 and TCF4, but not TCF1, by Wnt/HIPK2-dependent phosphorylation. Our data indicate that the physiological role for this phosphorylation is to decrease TCF binding to target promoters. Moreover, we find that this phosphorylation leads to the replacement of the TCF3 repressor with the TCF1 activator, revealing a novel "TCF switch" mechanism for transcriptional activation.

EXPERIMENTAL PROCEDURES

Plasmids—pCS2-FLAGTCF1 was derived from pT7TS-TCF1EC (10) by inserting the FLAG epitope using site-directed mutagenesis and subcloning into pCS2+ (48). For pCS2-FLAGLEF1 and pCS2-FLAGTCF4, the coding region of mouse LEF1 and *Xenopus* TCF4A was amplified by PCR from pGlomyc-mLEF1 (49) and pCS2-XTCF4A (50), respectively, and subcloned into pCS2FLAG.[3] Point mutants for pCS2FLAG-LEF1 were generated by using single primer-based site-directed mutagenesis. Constructs of TCF3, HIPK2, and *Vent2*-Luc[4] constructs were described previously (47). pCTX-mycHIPK2KD contains the substitutions $Lys^{221} \rightarrow$ Ala (in the catalytic lysine) and STY348–350 → AAF (in the activation loop). pCTX-mycHIPK2ΔPEST (HIPK2ΔP) lacks amino acids 841–867 and is unable to bind p53 and induce apoptosis (47). Primer sequences for PCR amplification and mutagenesis are shown in Table 1. Cloning and mutagenesis were verified by sequencing. Further details of cloning are available on request.

Xenopus Embryos and Microinjections—*In vitro* fertilization, embryo staging, and culture in 0.1× Marc's modified Ringer's solution were carried out as described (51, 52). Capped synthetic RNAs were generated by *in vitro* transcription using the mMESSAGE mMACHINE kit (Ambion) and the following linearized DNA templates: pCS2-Wnt8, pCS2-FLAG-β-catenin (53), pT7TS-HAXTCF3 (54), pCS2-FLAGTCF3HA, pCS2-FLAGTCF1, pCS2-FLAGLEF1, pCS2-FLAGTCF4, and pCTX-mycHIPK2. DNA injections involved pCS2+, pCS2-Wnt8, pCS2-dnWnt8 (55), pCS2-Wnt8myc (56), and *Vent2-Luc* (57). Other templates were as follows: *Xenopus* Wnt5a (56), mWnt7b, and mWnt2a in pCS2 (gifts of E. Morrisey), *Xenopus* Frizzled 8 (58), Ror2 (56), Ryk (59), mouse ΔRGS-Axin (60), rat GSK3, rat GSK3 K85R (61), LRP6, and LRP6–5m (62). For microinjections, embryos were transferred into 3% Ficoll 400 (Pharmacia) in 0.5× Marc's modified Ringer's solution and injected at the four to eight-cell stages with 10 nl of mRNA or DNA solution (63).

Immunoprecipitation, Western Analysis, and Alkaline Phosphatase Treatment—*Xenopus* embryos and HEK293T cells were lysed in 300–500 μl of buffer containing 0.5–1% Triton X-100, 50 mM Tris-HCl, 50–150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 10 mM NaF, 1 mM Na_3VO_4. Supernatants were cleared at 12,000 × *g* for 5 min and incubated with anti-FLAG-agarose beads (Sigma), 9E10 (anti-Myc), or anti-N-terminal XTCF3 (64) at 4 °C overnight. Protein A-Sepharose was used for 9E10 or anti-N-terminal XTCF3 antibodies. Antibody-bound beads were washed three times with lysis buffer and boiled in the SDS-PAGE sample buffer. For alkaline phosphatase treatment, antibody-bound beads were incubated in New England Biolabs buffer 3 with 0.5 units/ml of calf intestine phosphatase (New England Biolabs) for 40 min at room temperature. Monoclonal antibody 9E10, 12CA5, M2 (Sigma), and anti-VSVG (Sigma) antibodies were used for detection of Myc-, HA-, FLAG-, and VSVG-tagged proteins, respectively. Other antibodies were for nonphosphorylated β-catenin (ABC, Upstate Biotechnology), total β-catenin (Sigma), β-tubulin (BioGenex), and α-tubulin (Sigma).

In Vitro Immune Complex Kinase Assay—HEK293T cells were transfected separately with pCS2FLAGTCF3HA, pCS2FLAGTCF3P2/3/4HA, pCS2FLAGLEF1, pCS2FLAG-LEF1P2/3, pCS2FLAGTCF4, pCTXmycHIPK2FLAG, or pCTX-mycHIPK2KDFLAG. Cell lysates were combined, and proteins were coprecipitated with anti-FLAG-agarose beads. Immunoprecipitated proteins were incubated with the kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) with or without [γ-^{32}P]ATP (0.04 mCi/ml) at 30 °C for 45 min. The products of the kinase reaction were separated on SDS-PAGE gels and subjected to autoradiography and probed on Western blots with anti-FLAG antibody.

Chromatin Immunoprecipitation Assays—ChIP assays were carried out from *Xenopus* embryonic tissues as described (65–67) with modifications. Lysates of 30–50 injected embryos or explants were cross-linked with 1% formaldehyde for 30–60 min. The cross-linked samples were sonicated in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, and 0.5 mM dithiothreitol) with SONICS Vibra Cell™ sonicator. Anti-FLAG (M2)-agarose-beads (Sigma) were used to precipitate FLAGTCF proteins. After washing, protein complexes were reverse cross-linked at 65 °C overnight and treated with proteinase K. DNA fragments were purified with ChIP DNA Clean & Concentrator™ (ZYMO Research). Each experiment was reproduced 3–5 times. For PCR, the following primers were utilized to detect *Vent2* and *EF1a* genes: *Vent2* (forward) 5′-GGCAGACATGGTGGAGCCAG-3′ and (reverse) 5′-GTATGCAAATGCAGCCACTA-3′; *EF1a* (forward) 5′-ACAAAAGAGCTGGGAGCT-3′ and (reverse) 5′-TTCCTTTCCCATTGTGGA-3′.

[3] H. Hikasa, unpublished data.
[4] The abbreviations used are: Luc, luciferase; GSK3, glycogen synthase kinase 3; BRE, bone morphogenetic protein response element; MO, morpholino.

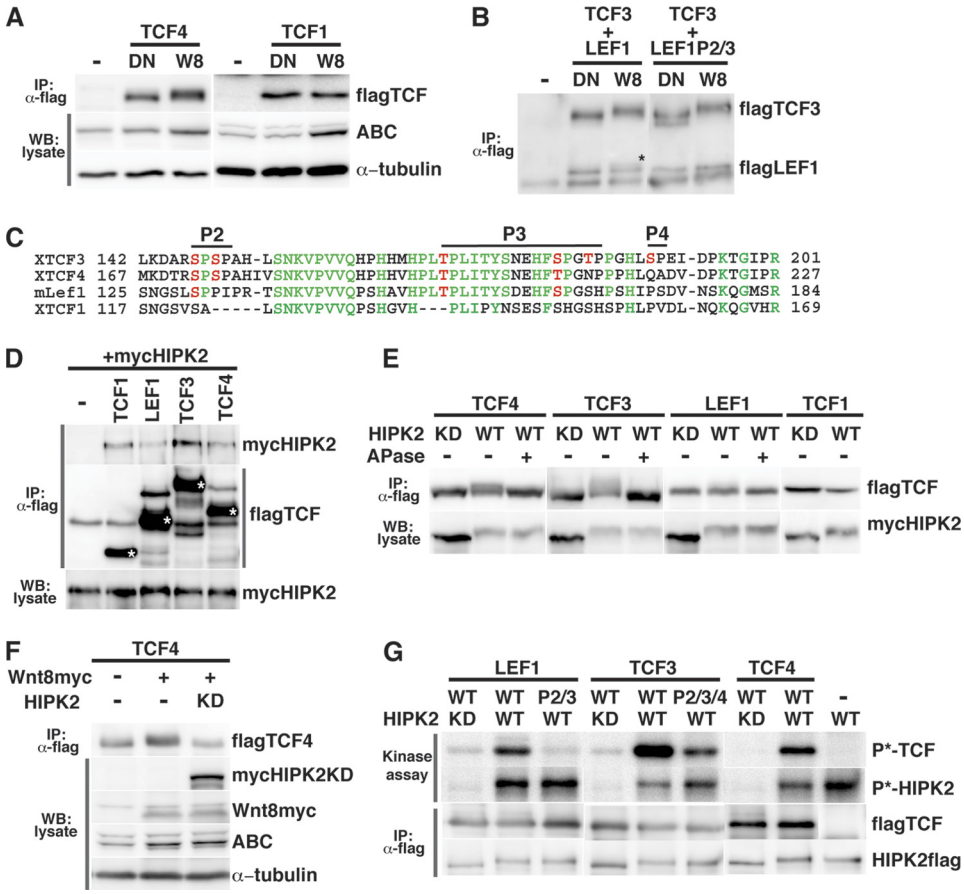

FIGURE 1. **TCF proteins are phosphorylated by Wnt8/HIPK2 stimulation.** *A* and *B*, TCF protein mobility is altered in response to Wnt8 stimulation. To express TCF proteins, FLAG-tagged TCF1, LEF1, TCF3, and TCF4 RNAs (2–4 pg each) have been microinjected into the animal pole of two-to-four cell stage *Xenopus* embryos together with Wnt8 (*W8*) or dominant negative Wnt8 (*DN*) DNA (100 pg each) as indicated. TCF3 and LEF1 RNAs were coinjected. Animal pole explants were isolated from the injected embryos at stage 8.5–9, and cell lysates were made at stages 12/13. TCF proteins were precipitated by anti-FLAG beads for Western analysis with antibodies to FLAG, unphosphorylated β-catenin (*ABC*), and α-tubulin (loading control). *A*, TCF4, but not TCF1, reveals a mobility shift in Wnt8-stimulated cells. *B*, LEF1 (*asterisk*) and TCF3 reveal a mobility shift in response to Wnt8. No change in mobility is observed for LEF1P2/3 carrying alanine substitutions at the P2 and P3 sites, indicating that these sites are responsible for the observed phosphorylation. *C*, the alignment of sequences containing putative phosphorylation sites in different TCF proteins. Conserved proline-directed Ser/Thr residues are highlighted in red. The following Ser/Thr residues were substituted for alanine in TCF3 and LEF1: *P2* (Ser^{147} and Ser^{149} for TCF3; Ser^{130} for LEF1), *P3* (Thr^{170}, Ser^{181}, and Ser^{184} for TCF3; Thr^{153} and Ser^{164} for LEF1), *P4* (Ser^{190}). *D* and *E*, HIPK2 binds (*D*) and phosphorylates (*E*) different TCF proteins. 293T cells were cotransfected with WT or kinase-dead (*KD*) Myc-HIPK2 (5 μg) and FLAGTCF constructs (5 μg) and were analyzed with anti-FLAG antibodies as described in *A*. *D*, Myc-HIPK2 associates with immunoprecipitated (*IP*) TCF1, LEF1, TCF3, and TCF4 (*white asterisks*). *E*, gel mobilities of all TCF proteins, except TCF1, are altered in HIPK2-expressing cells, and this shift was prevented by alkaline phosphatase (*APase*) treatment. *F*, HIPK2 is required for TCF4 phosphorylation in response to Wnt8. Dominant negative HIPK2 (*HIPK2KD*, 300 pg) blocks TCF4 phosphorylation in ectoderm explants (stage 12) from embryos injected with Wnt8myc DNA (100 pg). *G*, *in vitro* immune complex kinase assay. HEK293T cells were separately transfected with FLAGTCF constructs (10 μg) and WT or kinase-dead HIPK2 (5 μg). Cell lysates were prepared 24 h after transfections. Kinase reactions were performed with individual proteins precipitated with anti-FLAG beads in the presence or absence of [γ-^{32}P]ATP as indicated. Autophosphorylation of HIPK2 and TCF protein phosphorylation are visible after autoradiography (*top panels*, *P**). Protein levels were assessed on Western blots with anti-FLAG antibody (*bottom two panels*). *WB*, Western blot.

Luciferase Reporter Assays—Two-cell stage embryos were animally or marginally injected with 20–30 pg of reporter DNA together with the indicated RNAs or MOs. *BREm* and *BREm/TCFm* reporter constructs have been described (47). At gastrula stages (stages 11–13), embryos were homogenized in 50 mM Tris-HCl (pH 7.5). Supernatants were cleared by centrifugation at 12,000 × *g* for 3 min and assayed for luciferase activity as described previously (68). Every experimental group included four samples, each comprising seven embryos. All luciferase assays were repeated at least three times.

Cell Culture and Transfection—HEK293T were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Gemini-Bioscience) and penicillin/streptomycin (Sigma). HEK293T were plated for transfection the next day using linear polyethylenimine (molecular weight, 25,000; Polysciences) as described (69) with modifications (70).

RESULTS

TCF Proteins Are Phosphorylated by HIPK2 in Response to Wnt8 Stimulation—Given the importance of TCF regulation for Wnt signaling, we examined the mobility of different TCF proteins in response to Wnt8 in *Xenopus* embryos. We observed that gel mobilities of LEF1, TCF4, and TCF3, but not that of TCF1, were altered in cells stimulated by Wnt8 (Fig. 1, *A* and *B*). To determine which LEF1 sites are phosphorylated, we noticed that LEF1 contains two of the three HIPK2 phosphorylation sites (P2 and P3) that we identified in TCF3 (Fig. 1*C*) (47). Gel mobility of the LEF1 mutant protein with alanine sub-

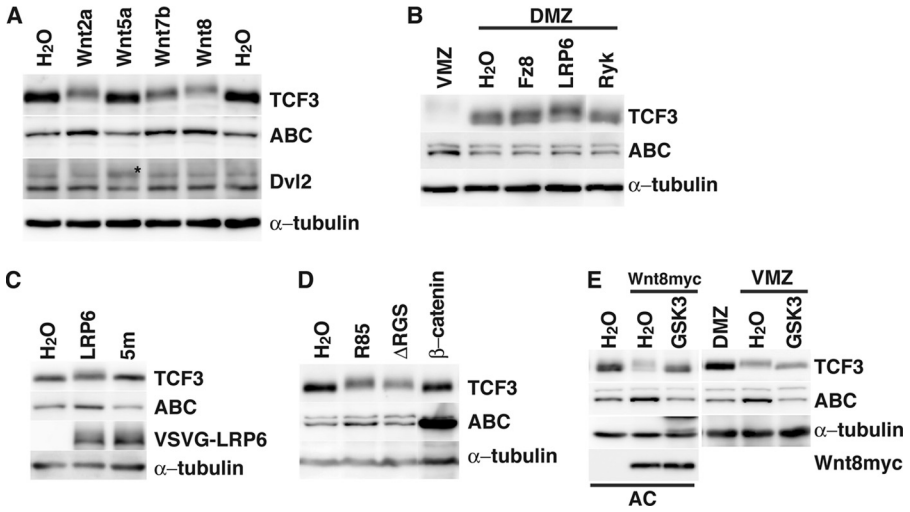

FIGURE 2. **TCF3 phosphorylation involves the LRP6/Axin/GSK3 pathway.** Two-to-four cell embryos were injected animally with RNAs encoding different Wnt ligands (*A*), receptors (*B* and *C*) or intracellular mediators of Wnt signaling (*D* and *E*). Animal caps were isolated at stage 8, cultured until stage 12. Cell lysates were prepared, and TCF3 mobility was assessed on Western blots using anti-TCF3 antibodies. Unphosphorylated β-catenin (*ABC*; *A–E*), and phosphorylated Dvl2 (*asterisk*; *A*) are shown to confirm Wnt signaling activity, α-tubulin is a loading control. Constructs were used at the following doses: Wnt2a, 7b, 5a, 8, and Wnt8myc DNAs (100 pg each); Fz8, Ror2, Ryk, LRP6, and LRP6 5 mRNAs (1 ng), β-catenin (50 pg), GSK3 and dominant negative GSK3 (R85) (0.5 ng each), and ΔRGSAxin RNA (1 ng). *C*, levels of tagged LRP6 proteins were controlled by anti-tag (VSVG) antibody. *DMZ*, dorsal marginal zone; *VMZ*, ventral marginal zone; *AC*, animal caps.

stitutions at both P2 and P3 sites (LEF1P2/3) was unaltered after Wnt8 stimulation (Fig. 1*B*), confirming that the observed shift in LEF1 is a result of specific phosphorylation at these sites.

Supporting our hypothesis that HIPK2 is involved in LEF1 and TCF4 phosphorylation. HIPK2 physically interacted with these TCF proteins in HEK293T cells (Fig. 1*D*), and its overexpression in HEK293T cells caused a mobility shift of LEF1 and TCF4 but not of TCF1 (Fig. 1*E*). This shift was reversed by alkaline phosphatase treatment (Fig. 1*E*), indicating that it is a result of phosphorylation. Moreover, a dominant negative form of HIPK2, inhibited Wnt8-mediated phosphorylation of TCF4 (Fig. 1*F*) as well as TCF3 (47), demonstrating that HIPK2 is required for this phosphorylation.

TCF protein phosphorylation by HIPK2 has been further confirmed by immune complex *in vitro* kinase assay with LEF1, TCF3 and TCF4 (Fig. 1*G*). In agreement with the lack of mobility change of Lef1 mutant in response to Wnt8 (Fig. 1*B*), specific phosphorylation mutants of LEF1 and TCF3 treated with HIPK2, exhibited significantly less incorporation of [γ-^{32}P]ATP than wild-type proteins (Fig. 1*G*). Together, these results are consistent with the hypothesis that HIPK2 phosphorylates TCF proteins at several Wnt-responsive phosphorylation sites.

Shared Upstream Components Involved in TCF3 Phosphorylation and β-Catenin Stabilization—To gain more insight into how Wnt proteins trigger TCF phosphorylation, we examined the pathway leading to TCF3 phosphorylation. Wnt ligands are commonly divided into two groups based on their ability to stabilize β-catenin. Whereas Wnt1, -2, -3, and -8 can stabilize β-catenin, Wnt4, -5, and -11 are thought to signal in a β-catenin-independent manner (71). Previous reports indicated that LEF1 and TCF4 can be phosphorylated by Nlk (nemo-like kinase) in response to representatives of both groups of Wnt ligands: Wnt1 (28) and Wnt5a (72). We wanted to know which Wnt ligands and receptors are able to trigger TCF3 phosphorylation in *Xenopus* ectoderm. To this end, we used the TCF3 gel shift assay in lysates of animal cap (ectoderm) explants expressing different Wnt signaling components. We observed that Wnt2a, Wnt8, and Wnt7b but not Wnt5a stimulated TCF3 phosphorylation, which correlated with their ability to stabilize β-catenin (Fig. 2*A*). Wnt5a activity was confirmed by its ability to increase Dvl2 phosphorylation in *Xenopus* ectoderm (Fig. 2*A*). These results show that TCF3 phosphorylation is triggered by the canonical (β-catenin-stabilizing) Wnt proteins, but not "noncanonical" Wnt5a.

Consistent with these observations, LRP6, but not other Wnt receptors, including Frizzled 8, Ryk or Ror2, stimulated TCF3 phosphorylation (Fig. 2*B* and data not shown). In contrast, LRP6 mutant in PPPSP*X*P motifs that inhibit GSK3 activity (62, 73) failed to trigger TCF3 phosphorylation (Fig. 2*C*).

We next examined the intracellular Wnt signaling components and observed that both dominant negative forms of Axin (ΔRGS-Axin) (60) and GSK3-R85 (61), but not β-catenin, were able to stimulate TCF3 phosphorylation, with little if any effect of ΔRGS-Axin on β-catenin stability (Fig. 2*D*). Moreover, wild-type GSK3 blocked TCF3 phosphorylation in Wnt8-expressing ectoderm cells and ventral marginal zone explants containing highly phosphorylated TCF3 (Fig. 2*E*) (47). These findings demonstrate that GSK3 functions to inhibit TCF3 phosphorylation and indicate that the same upstream pathway components are involved in both β-catenin stabilization and TCF3 phosphorylation.

LEF1 and TCF1 Activate a Vent2 Reporter—Knockdown of LEF1 and TCF1 with specific morpholino oligonucleotides in zebrafish and *Xenopus* embryos resulted in deficient ventroposterior development, as opposed to TCF3 knockdowns that cause anterior defects (9, 10, 47, 74, 75). Despite these observations, the role of these TCF proteins in the regulation of specific Wnt target genes has not been clear. The *Vent2/Vent/Vox* genes (76 – 80) are relevant targets, which are expressed in the

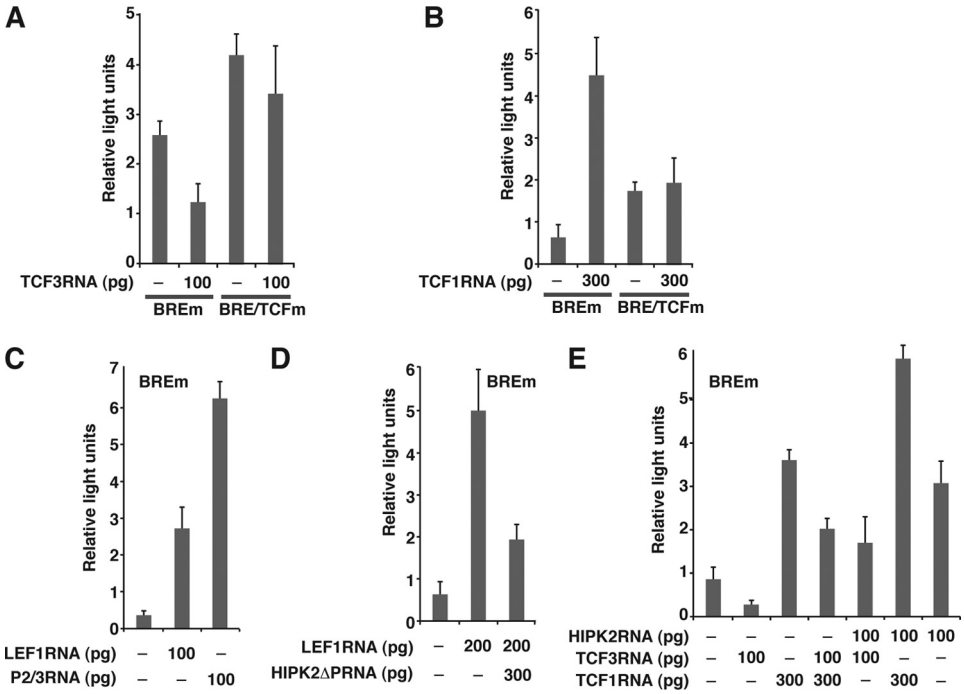

FIGURE 3. **Regulation of *Vent2* reporter activity by LEF1, TCF3, and TCF1.** Two-cell embryos were injected animally with *BREm-Luc* or *BRE/TCFm-Luc* reporter DNAs (20 pg each) and TCF1, LEF1, LEF1P2/3, TCF3, and HIPK RNAs as indicated and harvested at the late gastrula stage for luciferase activity determination. Each sample combines lysates from seven embryos. *BREm/TCFm* corresponds to *BREm* with point mutations in the unique TCF-binding site. Graphs are representative of triplicate experiments and shown as means ± S.D. with each group containing quadruplicate samples. *A*, TCF3 inhibits *BREm-Luc*, but not *BRE/TCFm-Luc*, reporter. *B*, TCF1 stimulates *BREm-Luc* through the unique TCF-binding site. *C*, LEF1 proteins activate *BREm-Luc*, with LEF1P2/3 being more potent transcriptional activator than wild-type LEF1. *D*, HIPK2 inhibits *BREm-Luc* reporter activation by LEF1. HK2ΔP is an active form of HIPK2 that lacks apoptosis-inducing activity (see "Experimental Procedures"). *E*, functional interaction of TCF3 and TCF1 in *BREm-Luc* reporter regulation. TCF1 and HIPK2 counteract the repressive effect of TCF3 on *BREm-Luc*.

ventrolateral embryonic region and can be activated by Wnt8 (81–84).

The *Vent2* promoter has been shown to contain two major regulatory elements, the bone morphogenetic protein response element (BRE) and a unique conserved TCF-binding site (47, 57, 85, 86). When BRE is mutated, such a reporter (BREm) is useful for Wnt signaling analysis in *Xenopus* gastrulae (47). However, roles of LEF1 and TCF1 in this system are unknown. To study the regulation of the *Vent2* gene by LEF1 and TCF1, embryos were injected with the *Vent2* reporter together with tagged TCF constructs, and luciferase activity was analyzed at late gastrula stages. Whereas TCF3 inhibited the reporter (Fig. 3*A*), we observed significant up-regulation of the reporter by LEF1 and TCF1, and this activation required the unique TCF-binding site (Fig. 3*B*, 3C). Interestingly, LEF1P2/3, insensitive to Wnt/HIPK2-mediated phosphorylation (Fig. 1*B*, 1F), more effectively activated *Vent2* reporter compared with wild-type LEF1 (Fig. 3*C*), indicating that HIPK2 inhibits LEF1 function by phosphorylation. We conclude that, in contrast to TCF3 that acts as a repressor, LEF1 and TCF1 function as transcriptional activators of the *Vent2* gene. This conclusion has been further confirmed in functional coinjection experiments (Fig. 3*E*, see below).

HIPK2 Dissociates TCF Proteins from Target Promoters—Because LEF1 behaves as a transcriptional activator (6, 9, 10), whereas TCF3 functions as a repressor (47, 75, 87, 88), HIPK2-dependent phosphorylation might have different consequences on transcription controlled by different TCF proteins. Supporting this hypothesis, HIPK2 inhibits LEF1-dependent reporter activation (Fig. 3*D*) but positively regulates TCF3-dependent transcription by antagonizing the function of TCF3 (Fig. 3*E*) (47). Our recent finding that unphosphorylated TCF3 mutants are more efficient transcriptional repressors than wild-type TCF3 (47) is also consistent with the idea that HIPK2 inhibits TCF3 function.

To explain the effect of HIPK2 on TCF function, we studied how HIPK2-mediated phosphorylation influences the association of LEF1, TCF3, and TCF4 with the *Vent2* promoter. Chromatin immunoprecipitation was used to assess occupancy of the *Vent2* promoter by tagged TCF proteins. We observed that the phosphorylation of LEF1, TCF3, and TCF4 by HIPK2 resulted in their removal from the *Vent2* promoter (Fig. 4, *A*–*C*). Our observations reveal a unified molecular mechanism for HIPK2-mediated phosphorylation, which causes the dissociation of TCFs from target DNA. These findings suggest that HIPK2 should function in the context-dependent manner, depending on the TCF protein that is present in the tissue of interest.

TCF Switching as a Possible Mechanism of Wnt Target Activation—Our previous results indicated that TCF3 represses the *Vent2* gene through a unique TCF site on the *Vent2* promoter (47). Given that Wnt proteins stimulate *Vent2* expression just by alleviating TCF3 repression, the mutation of the *Vent*2 promoter in the TCF-binding site should produce maximal reporter activity. However, upon coexpression of optimal doses of Wnt8 DNA, wild-type reporter was activated to a higher degree than achieved by mutagenesis of the TCF-binding site (Fig. 5*A*). This result implies that complete activation of

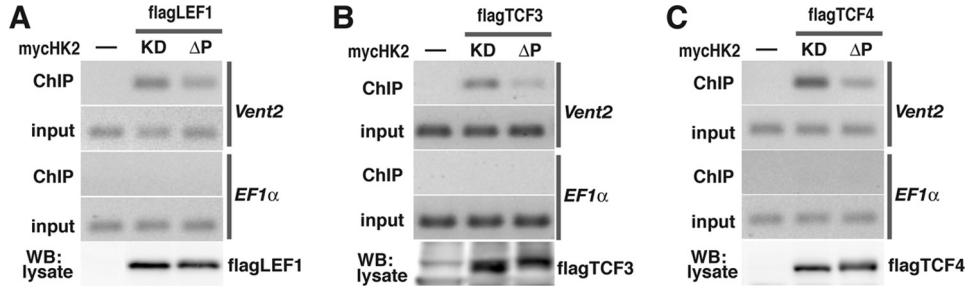

FIGURE 4. **HIPK2-mediated phosphorylation dissociates TCF proteins from promoter DNA.** Four-cell embryos were injected animally with TCF and HIPK2 RNAs as indicated and harvested at stage 12 for ChIP analysis. Doses of injected RNAs were as follows: TCF3 constructs, 30 pg; HK2KD, 400 pg; HK2ΔP, 400 pg. The ChIP assay was carried out with anti-FLAG antibodies as described (see "Experimental Procedures"). Active HIPK2 inhibits the binding of LEF1 (*A*), TCF3 (*B*), and TCF4 (*C*) to the *Vent2* promoter. *KD* is the kinase-dead form of HIPK2. Primers for EF1a were used as a control for specificity. TCF protein levels from the same experiment are shown. *WB*, Western blot.

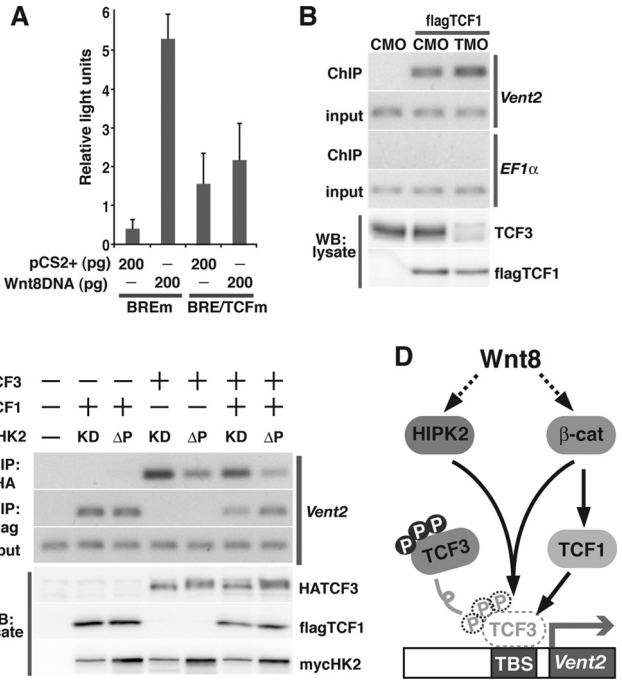

FIGURE 5. **HIPK2-mediated phosphorylation results in a substitution of TCF3 with TCF1 at the *Vent2* promoter.** *A*, *Vent2* reporter can be activated by Wnt8 to a higher degree than by mutations in the unique TCF-binding site. Two-cell embryos were injected animally with *BREm-Luc* or *BREm/TCFm-Luc* reporter DNA (20 pg each) and Wnt8 or control vector (pCS2) DNA (200 pg each). Luciferase activity was determined in embryo lysates at the late gastrula stage as described in Fig. 3 and under "Experimental Procedures." *B*, TCF1 occupancy of the *Vent2* promoter is increased after TCF3 depletion *in vivo*. Two-cell embryos were coinjected animally with FLAGTCF1 RNA (50 pg) and either TCF3 MO (*TMO*) or control MO (*CMO*) (20 ng each) and harvested at the late gastrula stage for ChIP analysis. The ChIP assay for TCF1 was carried out with anti-FLAG beads and *Vent2* promoter-specific primers. Primers for *EF1a* control specificity. Protein levels for this experiment are shown at the *bottom panel*. *C*, HIPK2 decreases TCF3, but enhances TCF1 occupancy of the *Vent2* promoter. Two-cell embryos were injected animally with FLAGTCF1 and HA-TCF3 RNAs (50 pg each) and HIPK2ΔP or the kinase-dead form of HIPK2 (*KD*; 300 pg each) as indicated, and harvested at the late gastrula stage for ChIP analysis. The ChIP assay was carried out with anti-FLAG for TCF1 and anti-HA for TCF3 (*top*). Primers for *EF1a* control specificity. Western blot (*WB*) shows protein levels for this experiment (*bottom*). *D*, TCF switch model of Wnt target stimulation. In the absence of Wnt8, TCF3 acts as a repressor of *Vent2* expression. After stimulation with Wnt8, TCF3 is phosphorylated by HIPK2 and dissociated from *Vent2* promoter. In place of TCF3, the β-catenin-TCF1 complex occupies the *Vent2* promoter and activates transcription.

Vent2 involves both elimination of TCF3-mediated repression and additional stimulation by a positively acting TCF protein.

To test this possibility, we studied the involvement of TCF1, which is expressed at the ventral margin and is required for ventroposterior development (9, 10). Moreover, TCF1 stimulated *Vent2* reporter via the same DNA binding site as used by TCF3 for repression (Fig. 3, *A* and *B*), suggesting a possible competition between TCF1 and TCF3 for the TCF-binding site. Reinforcing this hypothesis, TCF1 occupancy of the *Vent2* promoter has been elevated upon TCF3 knockdown with a specific, previously characterized morpholino oligonucleotide (Fig. 5*B*).

Xenopus TCF1 does not have the conserved P2/P3/P4 sites that are characteristic for TCF3 (Fig. 1*C*), and it does not show a mobility shift in response to Wnt8/HIPK2 signaling (Fig. 1*A* and 1*E*). In agreement with this lack of phosphorylation, TCF1 binding to the *Vent2* promoter did not change upon HIPK2 overexpression (Fig. 5*C*). Remarkably, co expression of HIPK2 with both TCF3 and TCF1 proteins weakened the association of TCF3 but enhanced the association of TCF1 with the *Vent2* promoter (Fig. 5*C*). Together, these observations demonstrate that, upon signaling, promoter occupancy switches from TCF3 to TCF1, supporting the idea that Wnt target genes are fully activated by the coordinated effect of the removal of TCF3-dependent repression and TCF1-mediated activation.

DISCUSSION

In this work, we analyzed the mechanism of TCF protein regulation by HIPK2 in *Xenopus* embryos. We find that Wnt stimulation or overexpression of HIPK2 cause phosphorylation of LEF-1, TCF4, and TCF3. This phosphorylation leads to the dissociation of TCF proteins from a target promoter and promotes gene target activation (in case of TCF3) or transcriptional repression (in case of LEF1). Because TCF proteins are the most downstream components of the signal transduction pathway from the cell surface to the nucleus, this regulation should be no less important than the control of β-catenin stability. Future studies will evaluate whether this phosphorylation is a diagnostic marker for cancers and whether it can serve as a basis of new drug screens.

The observed mechanism seems to be similar to the phosphorylation of POP1/TCF by the MOM-4/LIT1 kinase in *C. elegans* and mammalian TCF4 by the Wnt1/TGFβ-activated kinase/Nlk cascade in HEK293T cells, for which the upstream components are unknown (22, 25, 27–29, 89). Previous study reported that LEF1 is phosphorylated in response to Wnt5a/CamKII signaling (72), whereas we observed that TCF3 is phosphorylated by Wnt8/HIPK2 signaling (47). In this study, we found that TCF3 phosphorylation is triggered by canonical

Wnt ligands, LRP6, and dominant negative mutants for Axin and GSK3, but not β-catenin, indicating that TCF phosphorylation and β-catenin stabilization share the same upstream regulators. Interestingly, both Nlk and HIPK2 were reported to regulate c-Myb degradation in response to Wnt1 (42). Further experiments are required to better understand the relationship between the TGFβ-activated kinase/Nlk- and the canonical Wnt/HIPK2-mediated TCF phosphorylation.

Recent studies reached diverse conclusions regarding the role of HIPK in Wnt signaling. HIPK homologues were reported to suppress Wnt target gene expression in several experimental models (43, 44, 46), but positively regulate signaling in other models (45, 47). To explain the context-dependent function of HIPK proteins, one needs to consider that different TCF proteins are expressed in the spatially and temporally restricted fashion and have diverse roles in early development (9–11). According to our model, HIPK2 functions as a positive or negative regulator of Wnt signaling, depending on the functional properties of TCF proteins that are present in the embryonic tissue. Specifically, HIPK2 would inhibit the pathway when an activator type TCF, such as LEF1, is phosphorylated, but would activate it when phosphorylating the repressive form of TCF (TCF3). Indeed, HIPK2 stimulates a *Vent2* reporter by phosphorylating TCF3 (47) but inhibits LEF-1-dependent reporter activation by phosphorylating LEF1 (Figs. 3*D* and 4*A*). This observation is consistent with the study, in which the phosphorylation of the P3 site by Nlk was proposed to inhibit LEF1 activity (30). These findings are strongly supported by our data revealing the dissociation of different TCF proteins from the *Vent2* promoter (Fig. 4). There is no effect of HIPK2 on TCF1, consistent with the lack of the P2/P3/P4 phosphorylation sites in this gene. Together, our results provide a likely mechanism for the context-dependent effects of HIPK2 on Wnt signaling.

The regulatory mechanism for Wnt-dependent TCF phosphorylation differs significantly from the commonly accepted model of β-catenin/TCF coactivation of Wnt target genes, yet it has the same upstream regulators. It is unclear under which circumstances one branch is favored *versus* the other and whether the two branches might operate simultaneously. So far, we were unable to detect a change in HIPK2 enzymatic activity in embryos after Wnt stimulation (data not shown). We observed that at high doses of the Wnt signal, the *Vent2* reporter was activated to a higher degree than when the TCF3 repression was removed by TCF3 MO or when the TCF-binding site has been mutated (Fig. 5*A* and data not shown). This suggests the existence of an activation mechanism in addition to the derepression mechanism (Fig. 5*D*). This model is supported by our finding that upon HIPK2-mediated phosphorylation, TCF3 is replaced by TCF1 at the target promoter. Given that both TCF3 and TCF1 are co-expressed during gastrulation and function antagonistically (9, 10, 47), this mechanism is likely to operate during anteroposterior embryonic development.

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